



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : A61K 37/02, C07K 5/00	A1	(11) International Publication Number: WO 92/13549 (43) International Publication Date: 20 August 1992 (20.08.92)
(21) International Application Number: PCT/US92/00905 (22) International Filing Date: 4 February 1992 (04.02.92) (30) Priority data: 652,161 7 February 1991 (07.02.91) US (71) Applicant: RESEARCH CORPORATION TECHNOLOGIES, INC. [US/US]; 6840 East Broadway Boulevard, Tucson, AZ 85710 (US). (72) Inventors: HATHAWAY, David, R. ; 7966 N. Illinois Street, Indianapolis, IN 46260 (US). MARCH, Keith, L. ; 113611 Springmill Blvd., Carmel, IN 46032 (US).	(74) Agent: SCOTT, Anthony, C.; Scully, Scott, Murphy and Presser, 400 Garden City Plaza, Garden City, NY 11530 (US). (81) Designated States: AT (European patent), BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), MC (European patent), NL (European patent), SE (European patent). Published <i>With international search report.</i>	
(54) Title: INHIBITION OF CELL PROLIFERATION BY HYDROPHOBIC PEPTIDES (57) Abstract <p>The present invention is directed a method of using certain hydrophobic peptides for the inhibition of cell proliferation, wherein the peptides have the general formula: R-Xaa₁-(Xaa)_m-Xaa_n. The subject peptides have 2-7 amino acids such as alanine (Ala), arginine (Arg), cysteine (Cys), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), norleucine (nLeu), phenylalanine (Phe), proline (Pro), threonine (Thr), tyrosine (Tyr), tryptophan (Trp), or valine (Val). In accordance with the present invention, these peptides are potent inhibitors of cell proliferation as well as inhibitors of the synthesis of two cellular proto-oncogenes. One aspect of the present invention provides for the prevention and treatment of cancer by administration of the subject peptides. A further aspect of the present invention provides for inhibiting cell proliferation using the subject peptides in the treatment and prevention of prostatic hypertrophy, arterial occlusion (restenosis), arteriosclerosis and smooth muscle cell diseases.</p>		

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INHIBITION OF CELL PROLIFERATION
BY HYDROPHOBIC PEPTIDES

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Inappropriate, inopportune or excessive cell proliferation is a major cause not only of cancer but also of heart disease. Typically a cancerous cell loses its ability to respond to cellular signals for growth restraint. Similarly, unchecked smooth muscle cell growth within the inner lining of arteries is a major cause of arteriosclerosis and of reocclusion after arterial dilation.

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The present invention is directed towards the use of certain hydrophobic peptides to inhibit cellular growth, especially of smooth muscle cells and cancerous cells. Generally these hydrophobic peptides have 2-7 amino acids such as alanine (Ala), arginine (Arg), cysteine (Cys), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), norleucine (nLeu), phenylalanine (Phe), proline (Pro), threonine (Thr), tyrosine (Tyr), tryptophan (Trp) or valine (Val). In accordance with the present invention, these peptides are potent inhibitors of cell proliferation.

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One aspect of the present invention provides for the prevention and treatment of cancer by administering the subject peptides to an animal.

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Another aspect of the present invention relates to inhibiting cell proliferation of smooth muscle cells with the subject peptides. An additional aspect of this invention relates to the prevention of arterial occlusion in vivo using the subject peptides and methods of administration of these peptides for the prevention and treatment of arteriosclerosis.

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-2-

1 Biologists have long recognized that control of
cell proliferation is one of the most basic aspects of
multicellular existence. Throughout embryological
development, and through all of adult life,
5 differentiated cells have a choice of whether to divide
or not. Only if a programmed series of correct decisions
is made can the organism continue to function normally.
If incorrect decisions are made one cell type can
replicate unchecked, thereby interfering with critical
10 life functions and ultimately threatening the existence
of the organism.

Intertwined with any discussion or study of
cell proliferation is the nature and basis of cancer.
This collection of horrific diseases by definition
15 involves cells which divide when they should not, thus
producing tumors. Cancer can arise by changes in
differentiated cell types, resulting in cancer cells that
exhibit many of the morphological and functional
characteristics of their respective non-cancerous
20 precursor cells. Cancer is common in cells that normally
undergo frequent division (e.g. epithelial cells of the
skin) and so the problem may not be that cancer cells
divide frequently but that they lack the normal control
systems to stop unwanted cell division. Hence, in an
25 effort to find effective drugs for cancer therapy,
reagents are sought which control or inhibit cell
proliferation. The peptides of the present invention can
provide this control.

However, cancer is not the only disease that is
30 exacerbated by excessive cell proliferation. Researchers
also recognize that unchecked proliferation of smooth

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1 muscle cells can lead to partial blockage of arteries,
causing arteriosclerosis. Even after treatment of
arteriosclerosis by balloon dilation (angioplasty) of
5 blocked portions of an arteries, further smooth muscle
cell proliferation can cause reocclusion (restenosis) of
the artery. Hence, inhibition of smooth muscle cell
proliferation in arteriosclerosis, or after dilation of
arteries, provides a promising new treatment strategy.

Arteriosclerosis is a disease of the inner
10 lining, or intima, of arteries leading to formation of
fatty lesions on the arterial inner surfaces. The
earliest stage in the development of these lesions is
believed to be damage to the endothelial cells and
sublying intima. Damage can be caused by physical
15 abrasion of the endothelium, by abnormal substances in
the blood, or even by the effect of the pulsating
arterial pressure on the vessel wall. Once the damage
has occurred, smooth muscle cells proliferate and migrate
from the media (middle layer) of the arteries into the
20 lesions. Soon thereafter, lipidic substances, especially
cholesterol, begin to deposit within the proliferating
muscle cells, generating plaques. In later stages,
synthesis of extracellular matrix by fibroblasts and
other cells infiltrating the degenerative areas causes
25 progressive sclerosis (fibrosis) of the arteries.
Calcium often precipitates with lipids to generate
calcified plaques. When fibrosis and calcification
occurs, the arteries become extremely hard. The hardened
arteries lose most of their distensibility, and the
30 region within and surrounding them is easily ruptured.
Arteriosclerotic plaques often protrude through the

-4-

1 intima into the flowing blood, and the biochemical or
physical changes of the plaque surface can cause blood
clots to develop.

5 Almost half of all human beings die of arterio-
sclerosis. Approximately two thirds of these deaths are
caused by clot formation in a coronary artery, the
remaining one third occur by clot formation, or
hemorrhage of vessels, in other organs of the body,
especially in the brain (causing strokes).

10 A commonly used treatment for coronary arterio-
sclerosis is percutaneous transluminal coronary
angioplasty, or arterial dilation. This procedure is a
treatment of choice because it can enlarge a narrowed
arterial passageway. However, arterial injury may occur
15 during angioplasty and this injury can re-initiate or
intensify the process of plaque formation leading to
reocclusion of the artery. In fact reocclusion
(restenosis) of the artery appears to be an exaggerated
response to the controlled injury of angioplasty, and
20 occurs in 30-40% of all patients receiving angioplasty.
The process of restenosis is very similar to the
formation of the original arteriosclerotic plaque, but
occurs on a shorter time scale. Endothelial cell injury
caused by angioplasty leads to platelet aggregation and
25 shortly thereafter to activation of smooth-muscle cell
proliferation. Platelets secrete platelet-derived growth
factor (PDGF), which is one of the most potent cell
proliferative factors for smooth muscle cells found in
serum. PDGF is also a chemotactic attractant for smooth
30 muscle cells and may be responsible for attracting
smooth muscle cells from the middle layer, or media, of

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1 the artery into the intima as well as for the initial
proliferation of smooth muscle cells within the intima.
However, it is thought that PDGF alone does not optimally
stimulate DNA synthesis. PDGF-stimulated cells require a
5 second group of growth factors (termed "progression
factors"), to initiate DNA synthesis and cell division.
PDGF alone appears to simply stimulate cells to enter a
new cell cycle by causing the cell to move from the G_0
arrest state to G_1 (Fig. 1 depicts the time course of a
10 typical mammalian cell cycle). Exposure to progression
factors allows cells to move through the cell cycle by
initiating DNA synthesis (S phase). A number of
progression factors are known, including epidermal growth
factors from platelets and somatomedin-C present in
15 serum.

In accordance with the present invention,
specific peptides are shown to be effective in the
inhibition of cell proliferation, and provide new
reagents for the prevention and treatment of
20 arteriosclerosis, prostatic hypertrophy and various forms
of cancer. The block in cell division caused by the
present peptides appears to occur prior to the S, or DNA
synthesis, phase of the cell cycle. A secondary block to
cell division in the G_2 or M phase of the cell cycle, is
25 also observed when cells are exposed to the subject
peptides for longer periods of time.

The subject peptides are also inhibitors of
calcium-dependent thiol proteases, calpains I and II.
The calpains are cytosolic cysteine proteases which are
30 ubiquitously distributed in most cell types. The
biological function of calpains is not clear. However,

-6-

- 1 calpains have been implicated in numerous processes
including the re-structuring of the plasma membrane
(Zaidi, et al., 1989, J. Membrane Biol. 110:209-216), the
5 proteolysis of two important cytoskeletal proteins
(Yoshida, et al., 1984, FEBS Letters 170:259-262) as well
as the regulation of platelet aggregation (Fox, et al.,
1983, J. Biol. Chem. 258:9973-9981) and the activation of
several cellular regulatory proteins (Murachi et al.,
1981, in Advances in Enzyme Regulation, G. Weber, ed.,
10 Pergamon Press, New York, 19:407-424).

- The present invention is directed to a method
of inhibiting animal cell proliferation, especially the
inappropriate, inopportune, or excessive cell
proliferation associated with cancer, arteriosclerosis,
15 restenosis, and smooth muscle or endothelial cells. In
particular, the method provides for administering a
growth inhibiting amount of at least one of certain
hydrophobic peptides to an animal or to cultured cells,
wherein the subject peptides range from 2 to 7 amino acids
20 and are represented by the formula:



and further wherein

m is 0-5;

- 25 R is hydrogen, epoxysuccinyl, cholesteryl,
aryl, aralkyl or acyl;

- Xaa₁ and (Xaa)_m are independently Ala, Arg,
Ile, Leu, Lys, Met, nLeu, Phe, Pro, Thr, Tyr, Trp or Val;
Xaa_C is an amino acid from the group Ala, Arg,
Cys, Ile, Leu, Lys, nLeu, Phe, Pro, Thr, Tyr, Trp, Val or
30 the corresponding alcohol, aldehyde, epoxysuccinate, acid

-7-

- 1 halide, carbonyl halomethane or diazomethane derivative
of the carboxy terminal group of said amino acid.

Specifically, the preferred peptides of this
invention include shorter peptides with two or three
5 amino acids, i.e. m being 0 or 1, and more especially
include benzyloxycarbonyl-Leu-norleucinal and
acetyl-Leu-Leu-
norleucinal.

- Moreover, the present method is useful in
10 treating and preventing smooth muscle cell proliferation,
arteriosclerosis, restenosis, especially restenosis
occurring after percutaneous transluminal coronary
angioplasty, and cancer and cancerous-like conditions
such as prostatic hypertrophy, small cell carcinoma of
15 the lung and some endotheliomas and sarcomas.

Another aspect of the invention provides
pharmaceutical compositions containing the subject
peptides with a pharmaceutically acceptable carrier for
administration to an animal in accordance with the
20 methods of this invention.

Fig. 1 depicts the phases of the mammalian cell
cycle and the approximate duration of each phase.

Fig. 2 depicts the inhibitory effect of
calpeptin (benzyloxycarbonyl-leucine-norleucinal) on the
25 proliferation of vascular smooth muscle cells. The open
circles depict the growth of untreated cells; the solid
circles depict the growth of cells treated with
calpeptin.

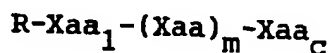
Fig. 3 depicts the normal distribution of DNA
30 in cultured vascular smooth muscle cells at various times

1 after addition of serum to serum depleted cells. At 12
hours (T=12 hr) after serum addition most cells have a 2n
DNA content (the amount of DNA normally found in resting
or non-dividing cells). Addition of serum allows the
5 cells to enter the cell cycle and to begin DNA synthesis.
By 24 hours (T=24 hr) after serum addition there is an
approximate equal distribution of cells with a 2n DNA
content and a 4n DNA content (twice the amount of DNA as
in non-dividing cells).

10 Fig. 4 depicts a comparison of the amount of
DNA in cells exposed to calpeptin with that in cells not
exposed to calpeptin. This figure demonstrates that
exposure to calpeptin arrests the division of most cells
prior to DNA synthesis, since most cells exposed to
15 calpeptin have a 2n content of DNA after 24 hours of
culture in the presence of serum. The top left panel
provides a control of dividing cells for comparison with
the other panels: a 2n DNA content is depicted by the
left peak and a 4n DNA content is depicted by the right
20 peak. Chronic exposure of cells to calpeptin allows some
progression through the cell cycle to the G₂ or M phase
of the cell cycle as demonstrated by a higher DNA content
in cells after 2 weeks exposure to calpeptin.

Fig. 5 depicts the effect that acetyl-Leu-Leu-
25 norleucinal (Inhibitor I), acetyl-Leu-Leu-methioninal
(Inhibitor II) and calpeptin have on DNA synthesis as
measured by ³H-thymidine incorporation in growing smooth
muscle cells. The amount of thymidine incorporated into
cells treated with 10⁻⁵ to 10⁻⁴M of calpeptin or
30 acetyl-Leu-Leu-norleucinal is significantly less than
that incorporated into control cells.

1 The present invention provides a method of
 inhibiting cell proliferation in vivo or in vitro, using
 a class of hydrophobic peptides which are effective for
 that purpose. A general formula depicting the structures
 5 of these peptides is:



wherein:

10 m is 0-5;

R is hydrogen, an epoxysuccinyl, a cholesteryl,
 aryl, aralkyl or acyl;

Xaa₁ and Xaa_m are independently Ala, Arg, Ile,
 Leu, Lys, norleucine (nLeu), Phe, Pro, Thr, Tyr, Trp, or
 15 Val;

Xaa_C is an amino acid from the group Ala, Arg,
 Cys, Ile, Leu, Lys, nLeu, Phe, Pro, Thr, Tyr, Trp, Val or
 the corresponding aldehyde, alcohol, epoxysuccinate, acid
 halide, carbonyl halomethane, or diazomethane derivative
 20 of the carboxy terminal amino acid.

Xaa₁ is the N-terminal amino acid; Xaa_C is the
 C-terminal amino acid and (Xaa)_m represents internal
 amino acids, if present. The values of m range from 0 to
 5, with preferred values of m being 0 to 3. The most
 25 preferred values for m are 0 or 1.

The aldehyde, alcohol, epoxysuccinate, acid
 halide, carbonyl halomethane, or diazomethane
 carboxy-terminal derivatives of Xaa_C are represented by
 the formulas -CH=O, -CH₂-OH, -CO-CH₂-CH-CO₂⁻, -CO-Y,
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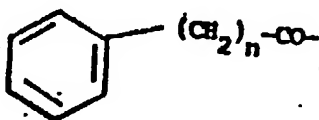
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1 -CO-CH₂-Y, or -CH-N₂ respectively. As used herein, Y is a
halo group, or halide, especially Cl, Br or I. In
particular, Cl or Br are preferred with Cl as the most
preferred.

5 When R is an acyl group it has the general
formula R'-CO, wherein R' is a lower alkyl or aryl group.

As used herein, the term lower alkyl refers to
alkyl groups containing one to six carbon atoms. These
groups may be straight-chained or branched and include
10 such moieties as methyl, ethyl, propyl, isopropyl,
n-butyl, sec-butyl, isobutyl, t-butyl, pentyl, amyl,
hexyl and the like. The preferred alkyl groups are C₁-C₄
alkyl.

When R or R' is an aryl group then the term
15 aryl, when used alone or in combination refers to an
aromatic ring containing six to ten carbon atoms.
Moreover, the present aryl groups include aralkyl groups
(aryl groups with lower alkyl groups as ring
substituents) and more specifically the groups benzyl,
20 benzoyl, naphthyl, carboxybenzyl, benzyloxycarbonyl, or



25 wherein n is an integer from 0-6 and preferably 0-3.
Aryl groups may have lower alkyl groups substituents at
any, some or all, available ring positions. Lower alkyl
group substituents are the same as those defined above.

30 Preferred R groups are cholesteryl,
benzyloxycarbonyl, acetyl or benzoyl groups. The most

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-11-

- 1 preferred R groups are acetyl, benzyloxycarbonyl and cholesteryl.

For Xaa₁ the preferred amino acids are Arg, Ile, Leu, Lys, Met, nLeu, Phe, Tyr or Val. The most
5 preferred are Leu nLeu or Val.

Each (Xaa)_m amino acid can independently be Ala, Arg, Ile, Leu, Lys, Met, nLeu, Phe, Pro, Tyr, Trp or Val. Preferred Xaa_m amino acids are Arg, Ile, Leu, Lys, Met, nLeu, Phe, Thr, Trp, Tyr, or Val. The most
10 preferred (Xaa)_m amino acids are Leu, nLeu, or Val.

Preferred Xaa_c amino acids are the aldehyde, alcohol, epoxysuccinate, acid halide, carbonyl halomethane or diazomethane derivatives of Arg, Ile, Leu, Lys, nLeu, Phe, Thr, Trp, Tyr or Val. The most preferred
15 carboxy-terminal amino acids are the aldehyde derivatives of Leu, Lys, nLeu, Phe or Tyr.

In particular, the preferred hydrophobic peptides of this invention are calpeptin (benzyloxycarbonyl-Leu-norleucinal) and acetyl-Leu-Leu
20 norleucinal.

The subject peptides may be chemically synthesized or isolated from a bacterial, fungal or plant source. Isolation can be by any technique used by one skilled in the art, including differential extraction,
25 ion-exchange or gel filtration column chromatographic procedures and high pressure liquid chromatography. Chemical synthesis of the subject peptides is by any of the methods for peptide synthesis, for example by either solution or solid phase synthetic procedures such as the
30 Merrifield procedure. Solid phase synthesis is commonly preferred for making longer peptides but many of the

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- 1 short peptides (with two to four amino acids) can be made
efficiently by solution synthesis.

5 A basic problem in peptide synthesis is one of
blocking or protecting the alpha amino group from
indiscriminate reaction with a carboxyl group of an
undesired amino acid; an additional problem is the
prevention of reactions with amino acid side chains.
These undesirable side reactions are prevented by use of
blocking groups that render an alpha amino group, or a
side chain group, unreactive but permit the desired
reaction to take place. In addition to providing
protection against undesirable reactions, the blocking
group must be easily removed without chemically altering
the remainder of the molecule, especially the peptide
linkage that has been built up during synthesis. (See
generally, Morrison and Boyd, Organic Chemistry, Third
Ed., Sec. 30.10 Synthesis of Peptides, pp. 1131-1133,
1983).

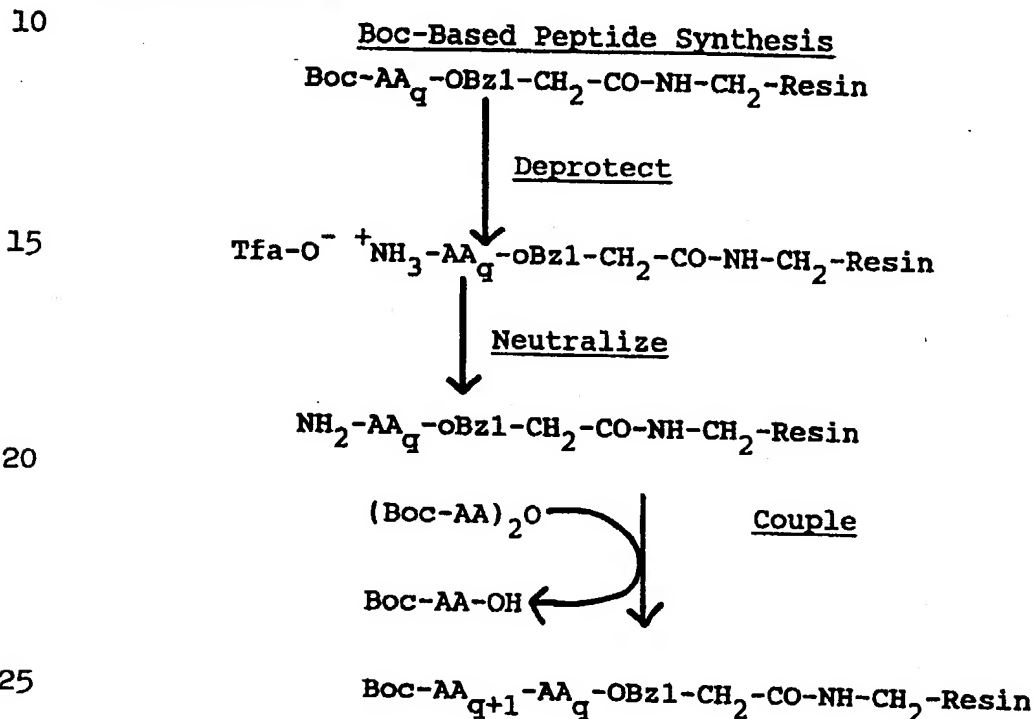
20 In practice, the strategy of maximal protection
of all side chain functionalities has been routinely
employed. There are at least two sets of side
chain-protecting groups and the choice of one set over
another is dictated by the protecting group strategy
chosen for the alpha amino group. General procedures for
peptide synthesis are provided in Barany et al. (1980, in
The Peptides 2: 1-284, Gross E. and Meienhofer, J. eds,
Academic Press, New York) and Stewart et al. (Solid Phase
Peptide Synthesis, Pierce Chemical Co.).

30 Commonly used alpha amino protecting groups are
tert-butyloxycarbonyl (Boc; cleaved by acid treatment),
9-fluorenylmethyloxycarbonyl (Fmoc; removed by treatment

-13-

1 with a secondary amine such as piperidine) and carbobenzyoxy (Z or Cbz; removed by catalytic hydrogenation).

5 The alpha-amino Boc, benzyl-based side chain protection strategy relies on the principle of graduated acid lability of the protecting groups. Boc-protected amino acids are inexpensive and available in high purity. A general scheme for Boc-based peptide synthesis is depicted below.



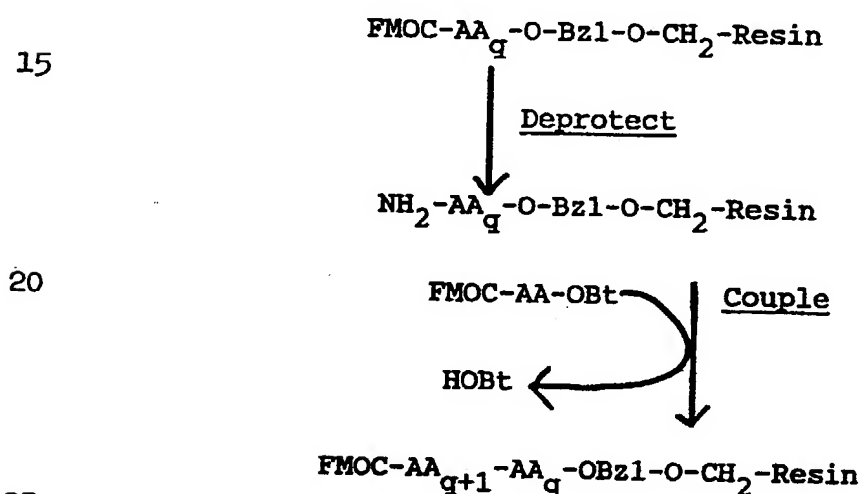
30 In the Boc-based peptide synthetic scheme depicted above, Boc-AA represents an amino acid with a Boc protecting group on its alpha amino group. The subscript q on AA depicts the number of amino acids in the peptide; q is an integer, from 1-6 in the present

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- 1 invention. Tfa-O^- is trifluoroacetic acid.
 OBzl- CH_2 -CO-NH- CH_2 -Resin represents a possible coupling
 arm and its attachment to a solid phase resin. As
 described above, use of a solid phase resin is optional,
 5 depending on the size of the peptide.

The Fmoc based strategy uses different
 mechanisms for removal of the alpha amino and side chain
 protecting groups: a secondary amine for the alpha amino
 protecting group and treatment with trifluoroacetic acid
 10 for the side chain protecting groups. An Fmoc-based
 peptide synthesis scheme is depicted below.

Fmoc-Based Peptide Synthesis

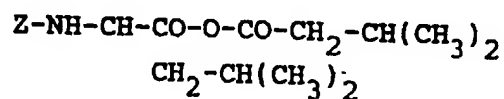
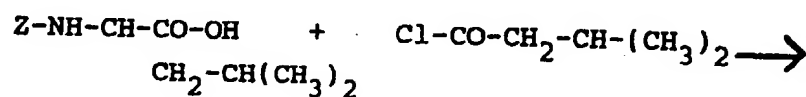


An Fmoc protected amino acid is represented by Fmoc-AA.
 The O-Bzl-O- CH_2 -Resin depicts an optional solid phase and
 its coupling arm to the synthetic peptide.

Synthesis of the present peptides need not be
 30 limited to the Boc and Fmoc synthetic techniques depicted
 above. For example benzyloxycarbonyl-leu-norleucinal

-15-

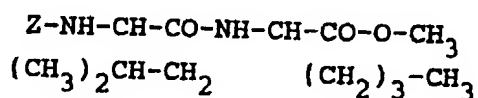
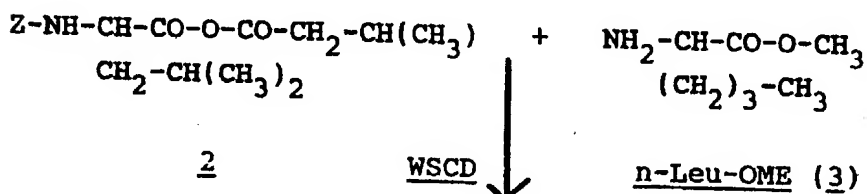
- 1 (calpeptin) may be synthesized in solution, using
 benzyloxycarbonyl-leucine (Z-Leu-OH, 1) as starting
 material. Z-Leu-OH is first reacted with
 isobutylchloroformate to make a mixed anhydride as
 5 depicted below.



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Mixed Anhydride (2)

- The mixed anhydride (2) is then coupled with the methyl
 ester of norleucine (n-Leu-OMe, 3) using a coupling
 20 reagent, for example, N-ethyl-N',N'-dimethylaminopropyl
 carbodiimide (WSCD).



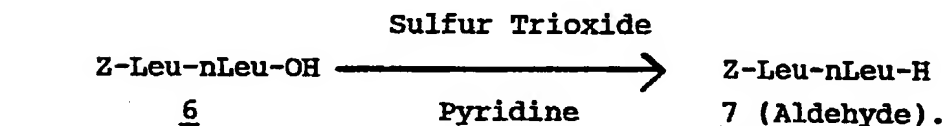
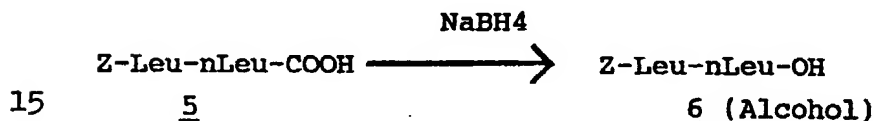
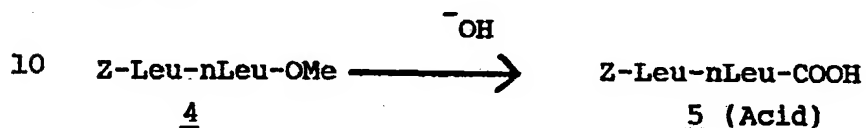
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Z-Leu-nLeu-OMe (4)

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-16-

- 1 The ester, Z-Leu-nLeu-OMe(4), can be saponified by
reaction with a strong base to form the acid (5).
Reduction with, for example sodium borohydride, yields
the corresponding alcohol (6) and subsequent partial
5 oxidation with sulfur trioxide pyridine complex,
triethylamine and dimethylsulfoxide, generates the
corresponding aldehyde (7). These reactions are depicted
below:



- The above synthetic reactions illustrate
general and specific techniques for synthesizing
peptides, and for generating the aldehyde or alcohol
derivatives from the C-terminal carboxylic acids.
- 25 Substituting the C-terminal carboxylic acid for
diazomethane can be accomplished by first converting the
carboxylate group into a mixed anhydride using, for
example, isobutyl chloroformate in tetrahydrofuran and
N-ethyl morpholine. This reaction mixture can then be
30 added to ethereal diazomethane and allowed to react
overnight. The diazomethane derivatized peptide may then

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- 1 be extracted with water and isolated. Chloromethane
derivatized peptides may be made from the diazomethane
derivative by treatment of the later with HCl in ethanol
at 0°C (Green et al., 1981, J. Biol. Chem. 256:
5 1923-1928; Sasaki, et al., 1986, J. Biochem. 99:
173-179).

In some cases the alpha amino group of the
subject peptide may be amidated by addition of a R'-CO-
group, e.g., acyl group, or an epoxysuccinyl group. This
10 can be done before, during or after peptide synthesis.
Amidation may be accomplished by any of the art
recognized procedures. For example, amide formation
frequently involves acylation of the amine with acid
chlorides, anhydrides, esters or carboxylic acids. The
15 reaction with carboxylic acids may be of limited utility
unless the acid is first activated so the -OH group
becomes a good leaving group. Carbodiimides can be used
to activate the carboxylic acid. Common acylating
procedures for amide formation employ acid chlorides or
20 anhydrides in pyridine.

Addition of a cholesteryl or an aryl group to
the N-terminal amino acid is most easily done before the
peptide is synthesized. This also prevents unwanted side
reactions with non-N-terminal amino acid side groups.
25 Blocking groups may be used on the N-terminal amino acid
side group and carboxylate, if needed. By using the
blocking groups described for use in peptide synthesis,
the cholesteryl- or aryl-derivatized N-terminal amino
acid may then be used directly in peptide synthesis.
30 Cholesterol or any aryl group may be derivatized at a
reactive position to provide a good leaving group. For

1 example, the C-3 alcohol of cholesterol may be
derivatized with p-toluenesulfonyl chloride
(p-CH₃-C₆H₄-SO₂Cl), thionyl chloride (SOCl₂) or be
5 replaced with a halide or other group to provide a
leaving group. Aryl groups with appropriate leaving
groups are frequently commercially available. The
appropriately blocked amino acid is then reacted with the
derivatized cholesterol or aryl group under conditions
10 which allow the leaving group to be replaced by the
N-terminal amine.

After synthesis, peptides may be purified and
salts may be removed by gel electrophoresis, flash
chromatography with a silica gel column, or by other size
selective chromatography procedures, including gel
15 chromatography or high-pressure liquid chromatography.

The present invention is directed to peptides
of the instant general formulae which have utility to
inhibit undesired cellular growth. Hence these peptides
are useful, for example, in the therapy or prevention of
20 cancer, for prevention or treatment of arterial occlusion
(arteriosclerosis) and for inhibiting smooth muscle or
endothelial cell proliferation.

Accordingly, the present invention provides a
method of inhibiting animal cell proliferation by
25 administering an effective amount of at least one of the
hydrophobic peptides defined in accordance with this
invention. An effective amount is that amount which is
sufficient to inhibit cell growth or proliferation,
particularly in cells which are in an inappropriate or
30 excessive growth phase, or an amount to retard or prevent
the course of a disease state.

1 In particular, the present method is useful for
inhibiting cell proliferation whether it occurs in vivo
or in vitro. When this method is used for in vivo cell
inhibition, the method can include administering the
5 subject peptides to an animal such as mammals, e.g.
humans, monkeys, rabbits, mice, cows, or a veterinary
animal or farm animal such as cats, dogs, chickens,
turkeys, horses and the like. By in vitro cell
proliferation is meant the inhibition of cultured cells,
10 e.g. tissue culture cells or primary cell cultures
obtained from a patient. Hence the method of inhibiting
cultured cells can include administering, treating or
co-culturing such cells in the presence of the subject
peptide. For example, the peptides may be co-cultured
15 with cells continuously or for varying periods of time.

Similarly, the subject peptides can be
administered singly or in combination as dictated by the
exigency of the condition. One skilled in the art can
readily select and combine the subject peptides for
20 simultaneous administration if necessary or as required
by the circumstances of treatment.

The present method is particularly useful for
inhibiting smooth muscle or endothelial cell
proliferation. Consequently, this method provides a
25 means of treating and preventing diseases associated with
inappropriate, inopportune, or excessive cell
proliferation of smooth muscle or endothelial tissues.
In particular, such diseases include arteriosclerosis,
restenosis and prostatic hypertrophy. The treatment and
30 prevention of restenosis by the present method is

1 especially useful after percutaneous transluminal
coronary angioplasty.

Moreover, the present method is particularly
useful for inhibiting the proliferation of cancerous
5 cells in an animal or in cell culture. Hence, the method
provides a means of treating and preventing cancer and
cancer-like diseases, e.g. benign tumors, arising from
inappropriate, inopportune or excessive cell
proliferation.

10 The administration of the subject peptides can
be accomplished by any convenient route known to those
skilled in the art by providing the subject peptides in
an effective amount sufficient to inhibit cell
proliferation as required by the exigency of the therapy.
15 Routes of administration include oral, enteric,
parenteral, intravenous, intramuscular, intrapericardial,
intranasal and topical. The amount of peptide delivered
varies by route and can be determined by one skilled in
the art in accordance with the guidelines provided
20 herein, especially as relates to pharmaceutical
compositions.

The present peptides inhibit cellular
proliferation both in vitro and in vivo. Cultured cells
can be used to test for the effects of the present
25 peptides on the rate of cell division. The rate of cell
division can be assessed by exposing cells to the subject
peptides, then counting cell number as a function of time
and comparing to controls. Addition of the subject
peptides to the cell culture medium causes a significant
30 decrease in cell growth. Cells grown without the subject
peptides increase in number by almost 10-fold in four

-21-

1 days. Cells grown in the presence of the subject
peptides show little or no increase in number over a
similar time period.

5 The rate of DNA synthesis can be determined by
observing the incorporation of labeled nucleotide into
cellular DNA. A nucleotide may be radioactively labeled.
Alternatively, a labeled nucleotide may be a nucleotide
analog that is readily incorporated into DNA and is
detected by a second detector molecule. One example of a
10 nucleotide analog is bromodeoxyuridine (BUdr) which may
be detected by, for example, fluorescently labeled
antibodies directed against bromodeoxyuridine. Total DNA
content may be assessed, for example, by using propidium
iodide.

15 To assess which stage of the cell cycle is
affected by the present antiproliferative agents, cells
may be synchronized or reversibly arrested in the G_0 , or
non-growing stage, of the cell cycle. Synchronization
can be achieved by any of a number of reagents known to
20 one skilled in the art, or simply by replacement of the
culture medium with serum-free medium for 2 or more days.
To initiate synchronized cell growth the cells may be
rinsed with rich medium containing no cell cycle
arresting reagent and then allowed to grow in normal,
25 serum containing medium. To test the affect that the
present peptides have on the growth of synchronized
cells, the peptides may be added to the growth medium at
various times after removal of the cell cycle arresting
reagent or after addition of serum to the medium, i.e.,
30 after releasing the cell cycle arrest.

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-22-

1 To assess the cell cycle stage of the cells,
flow cytometry can be used to sort cells on the basis of
DNA content. Cells which have incorporated more than a
normal 2n amount of labeled or analog nucleotide can be
5 distinguished, counted and sorted from cells with only a
normal 2n DNA content. DNA content is a reflection of a
cell's stage in the cell cycle. A 2n DNA content is the
normal content for a cell which is not synthesizing DNA.
Hence a cell with a 2n DNA content may be a non-growing
10 cell, or a cell which has just divided but not yet begun
DNA synthesis for another round of cell division. A 4n
DNA content indicates that the cell has finished
duplicating its normal DNA content but has not yet
physically divided into two daughter cells. A DNA
15 content between 2n and 4n indicates a cell is in the
process of DNA synthesis.

Cells exposed to the subject peptides for short
periods of time do not progress through the DNA synthesis
(S) phase of the cell cycle and hence have a 2n DNA
20 content long after cell cycle synchronization. However,
when exposed to the present peptides for up to 2 weeks
some cells may undergo DNA synthesis (i.e., have a DNA
content greater than 2n) but do not undergo cell
division, indicating that the present peptides can cause
25 a further block in the cycle at the G₂ or M phase.

In a further analysis of the biochemistry of
cells exposed to the peptides of this invention, the
present invention demonstrates that transcription of two
known cellular proto-oncogenes, c-fos and c-myc, are
30 depressed relative to that observed in cells not exposed
to the present peptides. The c-fos and c-myc gene

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1 products are believed to act as transcription factors
having a role in transformation of a cell to a malignant
phenotype. Hence, the present peptides are useful not
only for depressing cell growth but also for depressing
5 the production of factors that can lead to malignant
cancer.

The active ingredients of the present
pharmaceutical compositions include the present peptides
which exhibit antiproliferative activity when
10 administered in therapeutic amounts from about 0.1 mg to
about 2000 mg per kg of body weight per day and
preferably in amounts of from about 1.0 to 100 mg per kg
of body weight. Localized administration of a 1.0 to
1000 micromolar solutions of the subject peptides is
15 preferred. One skilled in the art can adjust the dosage
regimen to provide the optimum therapeutic response. For
example, one daily dose may be administered or several
divided doses may be given and the doses may be
proportionally reduced or increased as indicated by the
20 exigencies of the therapeutic situation. The active
compound may be administered in a convenient manner such
as by an intravenous, intraparacardial, oral,
intramuscular, intradermal, or subcutaneous route. The
active compounds may also be administered parenterally or
25 intraperitoneally. Intravenous or intrapericardial
administration is preferred.

Depending on the route of administration, the
active ingredients of the subject pharmaceutical
composition may be coated in a material to protect the
30 ingredients from the action of enzymes, acids or other
natural products. Dispersions can be prepared in

1 glycerol, liquid polyethylene glycols, oils and in
mixtures thereof. Under ordinary conditions of storage
and use, these preparations may contain a preservative to
prevent the growth of microorganisms.

5 The pharmaceutical preparations suitable for
injection include sterile liposomal suspensions, aqueous
solutions or dispersions, as well as sterile powders for
extemporaneous preparation of injectable solutions or
dispersions. In all cases the preparation must be
10 sterile and must be fluid to the extent that it is easily
syringable. A preparation must be stable under the
conditions of manufacture and storage and must be
preserved against the contaminating action of
microorganisms such as bacteria and fungi. The carrier
15 can be a solvent or dispersion medium containing, for
example, water, ethanol, polyol (for example, glycerol,
propylene glycol, liquid polyethylene glycol, and the
like), suitable mixtures thereof, and vegetable oils.
The proper fluidity can be maintained, for example, by
20 the use of a coating such as lecithin, by the maintenance
of the required particle size in the case of dispersion
and by the use of surfactants. The prevention of
microbial action can be brought about by various
antibacterial and antifungal agents, for example,
25 parabens, chlorobutanol, phenol, sorbic acid, thimerosal,
and the like. In many cases, it is preferable to include
isotonic agents, for example, sugars or sodium chloride.

Preferred carriers are those which protect the
active compound against rapid elimination from the body,
30 such as controlled release formulations, including
implants and microencapsulated delivery systems.

-25-

1 Biodegradable, biocompatible polymers can be used, such
as polyanhydrides, polyglycolic acid, collagen and
polylactic acid.

5 Liposomal carriers are also contemplated as
preferred carriers by the present invention. In addition
to the subject peptides, liposome carriers may
incorporate within them agents which help target the
subject peptides to the appropriate cell type, e.g.
10 antibodies directed against membrane proteins found only
on a specific cell type. Liposomal formulations may be
prepared by dissolving appropriate lipids in an inorganic
solvent which is subsequently evaporated to generate a
thin film of dried lipid on the surface of a container.
Appropriate lipids may include stearyl phosphatidyl
15 ethanolamine, stearyl phosphatidyl choline, arachadoyl
phosphatidyl choline and cholesterol. An aqueous
solution of the active compound, with the desired
additional carriers or agents as described above, is then
introduced into the container. The container is then
20 swirled to free the lipids from the sides of the
container and to disperse lipid aggregates, thereby
forming the liposomal suspension. Methods for
preparation of liposomal formations will be apparent to
those skilled in the art.

25 Sterile injectable solutions may also be
prepared by incorporating the active compounds in the
required amount and in the appropriate solvent with
various of the other ingredients enumerated above,
followed by filter sterilization. Generally, dispersions
30 are prepared by incorporating the various sterilized
active ingredients into a sterile vehicle which contains

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- 1 the basic dispersion medium and the required other
ingredients (enumerated above). In the case of sterile
powders for the preparation of sterile injectable
solutions, the preferred method of preparation is vacuum
5 drying. This freeze-drying technique yields a powder of
the active ingredient with any additional desired
ingredients from the previously sterile-filtered
solution.

- When the present peptides are suitably
10 protected they may be orally administered, for example,
with an inert diluent or with an assimilable edible
carrier, or they may be enclosed in hard or soft gelatin
capsule. They may also be compressed into tablets, or
15 incorporated directly into a food which is part of the
diet. For oral therapeutic administration, the active
peptide may be incorporated with excipients and used in
the form of ingestible tablets, buccal tablets, troches,
capsules, elixirs, suspensions, syrups, wafers, and the
like. Such compositions and preparations should contain
20 at least 1% of active compound. The percentage of the
compositions and preparations may, of course, be varied
and may conveniently be between about 5% to about 80% of
the weight of the unit. The amount of active compound in
such therapeutically useful compositions is such that a
25 suitable dosage is obtained. Preferred compositions or
preparations according to the present invention are
prepared so that an oral unit dosage form contains
between about 1 μ g and 2000 mg of active compound.

- The tablets, troches, pills, capsules and the
30 like may also contain the following: a binder such as
gum tragacanth, acacia, corn starch or gelatin;

1 excipients such as dicalcium phosphate; a disintegrating
agent such as corn starch, potato starch, alginic acid
and the like; a lubricant such as magnesium stearate; and
5 a sweetening agent such as sucrose, lactose or saccharin
may be added or a flavoring agent such as peppermint, oil
or wintergreen, or cherry flavoring. When the dosage
form is a capsule, it may contain, in addition to
materials of the above type, a liquid carrier. Various
10 other materials may be present as coatings or to
otherwise modify the physical form of the unit dosage.
For instance, tablets, pills, or capsules may be coated
with shellac. A syrup or elixir may contain the active
compound, sucrose as a sweetening agent, methyl and
15 propylparabens as preservatives, a dye and flavoring such
as cherry or orange flavor. Of course, any material used
in preparing any dosage unit form should be
pharmaceutically pure and substantially non-toxic in the
amounts employed. In addition, the active compound may
20 be incorporated into sustained-release preparations and
formulations.

It is especially advantageous to formulate
parenteral compositions in dosage unit form for ease of
administration and uniformity of dosage. Unit dosage
form as used herein refers to physically discrete units
25 suitable as unitary dosages for the mammalian subjects to
be treated; each unit containing a predetermined quantity
of active material calculated to produce the desired
therapeutic effect in association with the required
pharmaceutical carrier. The specification for the novel
30 dosage unit forms of the invention are dictated by and
directly dependent on (a) the unique characteristics of

-28-

1 the active material and the particular therapeutic effect
to be achieved, and (b) the limitations inherent in the
art of compounding such an active material for the
prevention of disease in living subjects.

5 The principal active peptide ingredient, is
compounded for convenient and effective administration in
pharmaceutically effective amounts with a suitable
pharmaceutically acceptable carrier in dosage unit form
as hereinbefore disclosed. A unit dosage form can, for
10 example, contain the principal active compound in amounts
ranging from 1 ug to about 2000 mg. Expressed in
proportions, the active compound is generally present in
from about 1 ug to about 2000 per ml of carrier. In the
case of compositions containing supplementary active
15 ingredients, the dosages are determined by reference to
the usual dose and manner of administration of the said
ingredients.

As used herein, "pharmaceutically acceptable
carrier" includes any and all solvents, dispersion media,
20 coatings, antibacterial and antifungal agents, isotonic
and absorption delaying agents, and the like. The use of
such media and agents for pharmaceutical active
substances is well known in the art. Except insofar as
any conventional media or agent is incompatible with the
25 active ingredient, its use in the therapeutic
compositions is contemplated. Supplementary active
ingredients can also be incorporated into the
compositions.

The following examples further illustrate the
30 invention.

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EXAMPLE 1Materials and MethodsCell Culture

5 Bovine aortic smooth muscle cells were obtained
by outgrowth from medial explants of thoracic aortae of
cows within 4 hours of slaughter. Initial outgrowth as
well as standard maintenance growth was in DMEM with 10%
FBS added. Media were renewed every 2-3 days. All
growth was in a humidified incubator equilibrated with a
10 5% CO₂ atmosphere. Cultures were passaged immediately
prior to full confluence by brief exposure to HBSS
(Hank's Balanced Salt Solution) containing trypsin (0.5
mg/ml) and EDTA (0.5 mM); all experiments were performed
using cells of passage 7 or less. As a test of
15 viability, cells were counted and assessed for trypan
blue exclusion with a hemocytometer at each passage and
at selected times during time course experiments,
routinely showing >95% of the population to exclude
trypan blue. For most subcultures and all experiments
20 cells were plated at a density of 10,000 cells/cm²,
regardless of container.

These cells exhibited typical morphologic
characteristics of vascular smooth muscle in vitro
including a pattern of variably multilayered growth, and
25 demonstrated specific immunoperoxidase staining by a
monoclonal antibody selective for muscle α -actin (HHF-35)
which did not react with endothelial cells, and is known
not to stain fibroblasts (Tsukada, et al., 1987, Am. J.
Pathol. 127:51-60).

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1 [³H] Thymidine Incorporation

 [³H] thymidine incorporation was used to assess
cell growth. After obtaining cells as described above
they were seeded in 24-well microtiter plates and allowed
5 to attach overnight. The cultures were washed with PBS
and placed in serum-free medium, consisting of DMEM with
1 μ M insulin and 5 μ g/ml transferrin added, for a total
of 48-52 hours (more than one doubling time for actively
cycling cells). During the last 6 hours of this
10 incubation, agents were added at variable concentrations
in a carrier solution consisting of 1:1 EtOH: H₂O to a
final EtOH concentration of 0.5 mg per 100 ml.
Serum-free medium was then removed and replaced by DMEM
with 10% FBS using concentrations of agent and carrier
15 solutions identical to those in the serum-free medium.
[³H] thymidine was added 18 hours after serum repletion
to a concentration of 2 μ Ci/ml, and was incubated for 6
additional hours. At the end of the incubation, cells
were released from the wells and incorporated precursor
20 removed by washing with distilled water. Cell residues
were collected on glass mesh filter by a automated cell
harvester. Radioactivity was measured by liquid
scintillation spectroscopy.

Flow Cytometric Assays

25 Cells were plated, allowed to attach, and
placed in serum free media for 48-52 hours as described
above. Compounds to be tested were added at the
designated concentrations in the serum-free media
followed by serum-containing media, also as described
30 above. At the specified times after serum addition,
these cells were harvested by trypsin/EDTA, washed with

- 1 HBSS/5% BSA, pelleted, and resuspended in PBS containing
0.6% NP-40 and 0.1 mg/ml propidium iodide, to which was
added RNase to a final concentration of 2mg/ml. Flow
cytometric analyses were done using a Becton-Dickinson
5 FACScan wherein the exciting wavelength was at 488 nm and
the detecting wavelength at 585 nm. Events were gated on
a fluorescence-area vs.-width map to eliminate potential
clumped nuclei. Such events represented less than 3% of
the total in general.

10 Calpeptin Synthesis

- Calpeptin was synthesized using an adaptation
of the protocol described by Tsujinaka et al. (1988,
Biochem. Biophys. Res. Comm. 153:1201-1208). Briefly,
Z-leu-OH was reacted with isobutyl chloroformate to form
15 a mixed anhydride intermediate; this was then coupled to
nLeu-OMe-HCl, followed by saponification with 1 N NaOH to
yield Z-Leu-nLeu-OH. This was reduced to the alcohol
with sodium borohydride, and then partially oxidized to
the corresponding aldehyde with sulfur trioxide/pyridine.
20 The overall yield starting from the original components
was 26% after purification by flash chromatography with a
silica gel column using initially 20% then 30% ethyl
acetate in hexanes mixture. Following recrystallization
from hot hexanes, the melting point was 90-93°C. Purity
25 was confirmed by TLC in 1:1 ethyl acetate:hexanes.

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EXAMPLE 2The Present Peptides are Antiproliferative
Agents for Cells in Culture

Cells were cultured as described in Example 1.

5 To assess cell growth, either tritiated thymidine or a deoxynucleotide analog, bromodeoxyuridine (BUdr) was added to the medium; the incorporation of tritiated thymidine or of fluorescently labeled antibodies directed against bromodeoxyuridine, respectively, gave a measure
10 of cell proliferation. Addition of propidium iodide to the medium allowed determination of the total amount of DNA. Cells were sorted by flow cytometry to distinguish populations of cells with different DNA (i.e., in this example, BUdr) contents.

15 Addition to the medium of benzyloxycarboxyl-Leu-norleucinal at 100 μ M or acetyl-Leu-Leu-norleucinal at 50 μ M caused a significant decrease in cell growth. As depicted in Fig. 2, the number of cells normally increased almost 10-fold in 4
20 days after serum addition but addition of calpeptin (benzyloxycarbonyl-Leu-norleucinal) resulted in almost no increase in cell number.

Removal of serum from the medium was used to synchronize the cell cycle of cultured cells (Fig. 3),
25 thereby allowing an assessment of which phase of the cell cycle was affected by the compounds tested. As depicted in Fig. 1, cells normally require about 24 hr to progress through the cell cycle. After serum depletion almost all cells are arrested in the G_0/G_1 phase with a $2n$ DNA
30 content (a normal, non-dividing amount of DNA). As depicted in Fig. 3, untreated cells exhibit little

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1 increase in DNA content at 12 hr after addition of serum.
However, by 18 hr a significant number of untreated cells
have more than a 2n DNA content, indicating that DNA
5 synthesis is progressing. By 21 hr, approximate equal
numbers of cells have a 2n or 4n DNA content, while the
remaining cells have a DNA content between 2n and 4n. By
24 hr almost all untreated cells have a 4n or 2n DNA
content. Cells with a 4n DNA content are likely undergo
cell division shortly, while those with a 2n DNA content
10 may have just undergone cell division.

Fig. 4 depicts the effect of calpeptin on the
DNA content of cells. In the absence of calpeptin and 24
hr after serum addition to serum-depleted cells, more
cells have a 4n DNA than a 2n DNA content, indicating
15 that significant DNA synthesis is occurring in these
cells. However, cells exposed to calpeptin do not
progress through the DNA synthesis phase of the cell
cycle, as demonstrated by the 2n DNA content of almost
all calpeptin exposed cells in Fig. 4. By 2 weeks after
20 serum addition most non-exposed cells have a 2n DNA
content indicating that they have stopped growing. Many
calpeptin-exposed cells, on the other hand, have a 4n DNA
content at 2 weeks after serum addition, indicating that
some cell cycle progression has occurred, and that an
25 additional block has occurred in these cells at the G₂ or
M phase of the cell cycle. No increase in aneuploidy of
DNA content (over 4n) was observed relative to controls.
Cell counting demonstrated that no cell growth had
occurred in 2 weeks in cells exposed to calpeptin or
30 acetyl-Leu-Leu-norleucinal and that there were less than
20% nonviable cells at any time. The EC₅₀ of calpeptin

1 and acetyl-Leu-Leu-norleucinal is 56 and 14 μ M,
respectively.

Fig. 5 depicts the effects of different
concentrations of calpeptin, acetyl-Leu-Leu-norleucinal
5 (Inhibitor I of Fig. 5) and acetyl-Leu-Leu-methioninal
(Inhibitor II of Fig. 5) on DNA synthesis in
proliferating aortic smooth muscle cells as measured by
tritiated thymidine incorporation by smooth muscle cells.
Cells exposed to either calpeptin or acetyl-Leu-Leu-
10 norleucinal incorporate significantly less tritiated
thymidine than do control cells or cells exposed to a
synthetic peptide which is not an anti-proliferative
agent (Inhibitor II).

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EXAMPLE 3The Present Peptides Inhibit Transcription
of Proto-Oncogenes

5 By Northern analysis of total cellular mRNA
produced after addition of serum to serum depleted cells,
acetyl-leucine-leucine-norleucinal caused a 4-fold
decrease, relative to controls, in the expression of
c-fos and c-myc, known cellular proto-oncogenes. A
10 similar decrease in actin mRNA was observed upon addition
of acetyl-leucine-leucine-norleucinal. In all cases,
mRNA was obtained from the same number of cells. The
production of mRNA from adenine phosphoribosyl-
transferase (APRT), a housekeeping gene required for
general metabolic function in all cells, was used as an
15 internal standard against which different mRNA
preparations could be compared. No decrease in APRT mRNA
was seen in treated cells, relative to untreated cells.
Hence, these peptides not only inhibit cell proliferation
but also depress the synthesis of cellular oncogenes.

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EXAMPLE 4Prevention of Restenosis After Angioplasty
by the Present Peptides

5 Acetyl-Leu-Leu-norleucinal has been shown to
reduce proliferation of cells in culture (Example 2). To
demonstrate that acetyl-Leu-Leu-norleucinal and related 0
peptides can have a similar effect in vivo, and be useful
in the prevention of arteriosclerosis and restenosis
after angioplasty, arteriosclerotic rabbits were treated
10 with acetyl-Leu-Leu-
norleucinal immediately after angioplasty. Prevention of
restenosis after angioplasty was used as a test system
for prevention of arteriosclerosis because the underlying
mechanisms of restenosis are like those occurring during
15 arteriosclerotic plaque formation.

A 50 μ M solution of acetyl-Leu-Leu-norleucinal
was slowly injected into 7 arteriosclerotic rabbit
femoral arteries immediately following angioplasty. A
porous Wolinsky catheter was used for this injection to
20 slowly disperse the peptide into the artery for a period
of 45 sec. As a control, five contralateral arteries
were injected with the carrier solution containing no
acetyl-Leu-Leu-norleucinal (Control 1). As a further
control, 12 femoral arteries were treated only with
25 angioplasty (Control 2). All animals were sacrificed
after angiography, which was done 2 weeks after
angioplasty. Arteries were sectioned into 3mm segments,
stained with hematoxylin/eosin and elastin/trichrome and
morphometry was performed. Results were evaluated by
30 analysis of variance.

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1 Table 1 summarizes the results of the study.
Angiography showed that the luminal diameter of animals
treated with acetyl-Leu-Leu-norleucinal was larger than
in controls, i.e. there was a smaller decrease after
5 angioplasty in treated than in non-treated animals.
Similarly, histological measurements indicated that
treated animals had a larger lumen area than control
animals. Neointimal area was the same for all groups
($0.76 \pm 0.42 \text{ mm}^2$ for control 1 animals and 0.63 ± 0.31
10 mm^2 for control 2 animals; $p = 0.817$). However, the
neointima/media ratio was smaller in
acetyl-Leu-Leu-norleucinal treated animals due to an
increased medial thickness. These data demonstrate that
injection of these peptides reduces post-angioplasty
15 restenosis while preserving the medial layer.

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TABLE 1

Effect of Acetyl-Leu-Leu-norleucinal
(Inhibitor 1) on Restenosis after Angioplasty

5	Treatment after angioplasty	Decrease in arterial lumina (by angioplasty	Luminal area (by histology)	Medial Thickness
10	None	$0.83 \pm 0.36 \text{ mm}$	$0.54 \pm 0.26 \text{ mm}^2$	0.38 ± 0.05
15	Injection with Carrier Solution	$0.88 \pm 0.33 \text{ mm}$	$0.55 \pm 0.19 \text{ mm}^2$	0.45 ± 0.14
20	Injection with Inhibitor 1	$0.26 \pm 0.27 \text{ mm}$	$0.84 \pm 0.40 \text{ mm}^2$	0.76 ± 0.38
25	Statistical significance (check)	$p = 0.004$	$p = 0.113$	$p = 0.02$

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1 WHAT IS CLAIMED IS:

1. A method of inhibiting animal cell proliferation which comprises administering an effective amount of a hydrophobic peptide sufficient to inhibit cell growth, wherein said peptide has the formula:



wherein:

m is 0-5;

R is hydrogen, epoxysuccinyl, cholesteryl, aryl, aralkyl or acyl;

Xaa₁ and (Xaa)_m are independently an amino acid selected from the group consisting of Ala, Arg, Ile, Leu, Lys, Met, nLeu, Phe, Pro, Thr, Tyr, Trp and Val;

Xaa_c is an amino acid selected from the group consisting of Ala, Arg, Cys, Ile, Leu, Lys, nLeu, Phe, Pro, Thr, Tyr, Trp and Val, or the corresponding alcohol, aldehyde, epoxysuccinate, acid halide, carbonyl halomethane or diazomethane derivative of the carboxy terminal group of said amino acid.

2. The method of Claim 1 wherein m is 1.

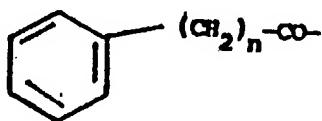
3. The method of Claim 1 wherein m is 0.

4. The method of Claim 2 or 3 wherein Xaa₁ is Leu, Val or nLeu.

5. The method of Claim 2 wherein Xaa_m is Leu or Val.

6. The method of Claim 2 or 3 wherein Xaa_c is Lys, Leu, nLeu, Phe or Tyr.

7. The method of Claim 2 or 3 wherein R is acetyl, benzyloxycarbonyl, cholesteryl, epoxysuccinyl, phenyl or



1 wherein n is 0 to 6.

8. The method of Claim 2 wherein the hydrophobic peptide is acetyl-Leu-Leu-norleucinal.

5 9. The method of Claim 3 wherein the hydrophobic peptide is benzyloxycarbonyl-Leu-norleucinal.

10. The method according to any one of Claims 1, 2, 3, 8 or 9 wherein said cell proliferation is inhibited in smooth muscle cells.

10 11. The method according to any one of Claims 1, 2, 3, 8 or 9 wherein said cell proliferation is inhibited for the treatment of prostatic hypertrophy.

12. The method according to Claims 1, 2, 3, 8 or 9 wherein said cell proliferation is inhibited for the treatment of arteriosclerosis.

15 13. The method according to any one of Claims 1, 2, 3, 8 or 9 wherein said cell proliferation is inhibited for the treatment of restenosis.

20 14. The method of Claim 13 wherein said restenosis follows percutaneous transluminal coronary angioplasty.

15. The method according to any one of Claims 1, 2, 3, 8 or 9 wherein said cell proliferation is inhibited for the treatment of cancerous cell proliferation.

25 16. A pharmaceutical composition for inhibiting cell proliferation comprising a therapeutic amount of at least one hydrophobic peptide and a pharmaceutically acceptable carrier, wherein said peptide has the formula:

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$$R-Xaa_1-(Xaa)_m-Xaa_C$$
and further wherein:

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m is 0-5;

R is hydrogen, epoxysuccinyl, cholesteryl, aryl, aralkyl or acyl;

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Xaa₁ and (Xaa)_m are independently an amino acid selected from the group consisting of Ala, Arg, Ile, Leu, Lys, Met, nLeu, Phe, Pro, Thr, Tyr, Trp and Val;

10

Xaa_c is an amino acid selected from the group consisting of Ala, Arg, Cys, Ile, Leu, Lys, nLeu, Phe, Pro, Thr, Tyr, Trp and Val, or the corresponding alcohol, aldehyde, epoxysuccinate, acid halide, carbonyl halomethane or diazomethane derivative of the carboxy terminal group of said amino acid.

15

17. The composition of Claim 16 wherein said peptide is acetyl-Leu-Leu-norleucinal or benzyloxycarbonyl-Leu-norleucinal.

20

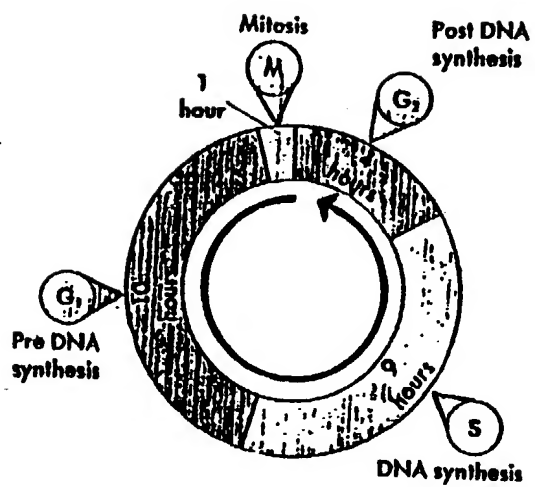
18. The composition of Claim 16 wherein said peptide is present in an amount to provide from about 0.1 mg to about 2000 mg per kilogram of body weight per day.

25

30

35

Figure 1



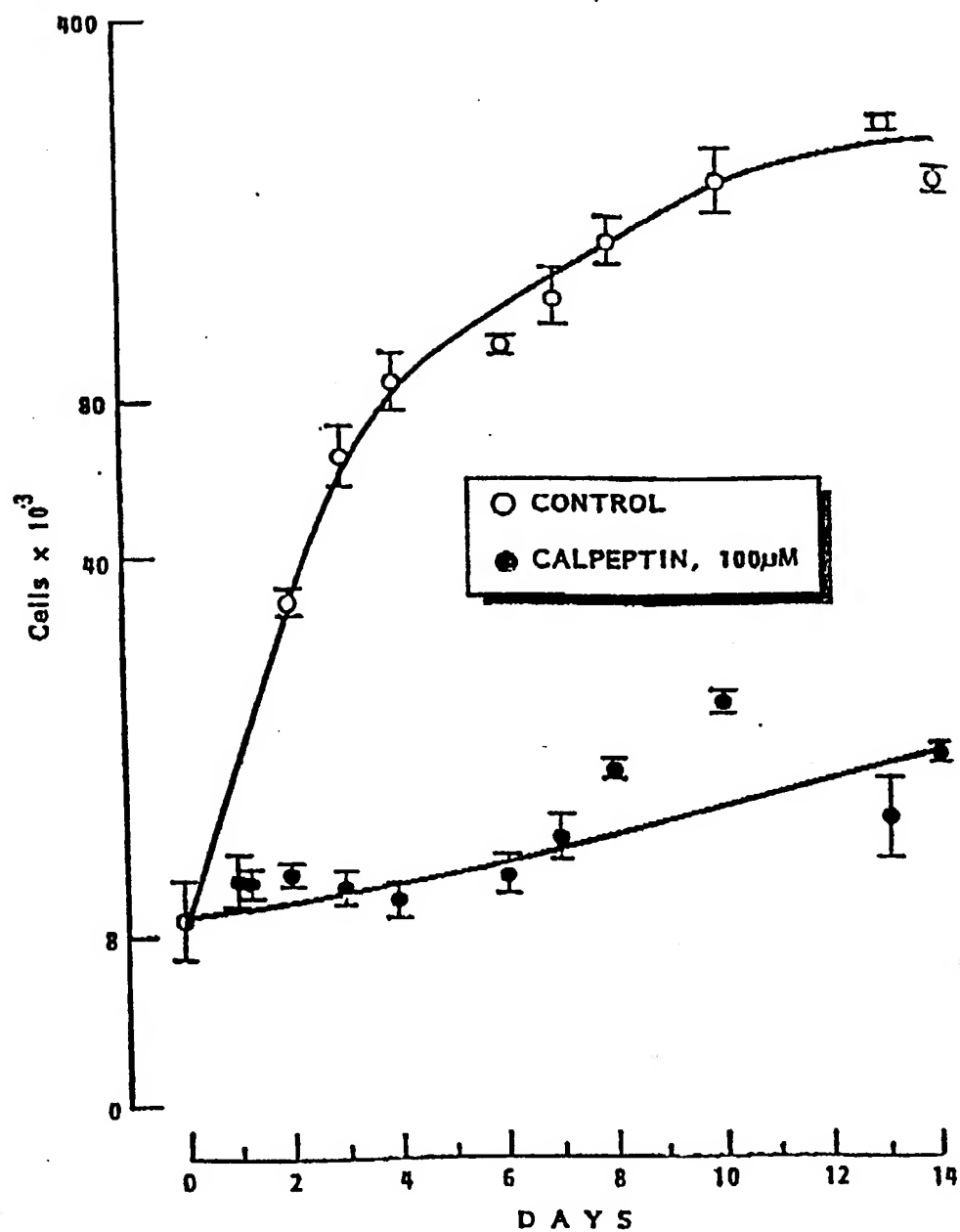
Effects of Calpeptin on Proliferation of
Vascular Smooth Muscle Cells

Figure 3

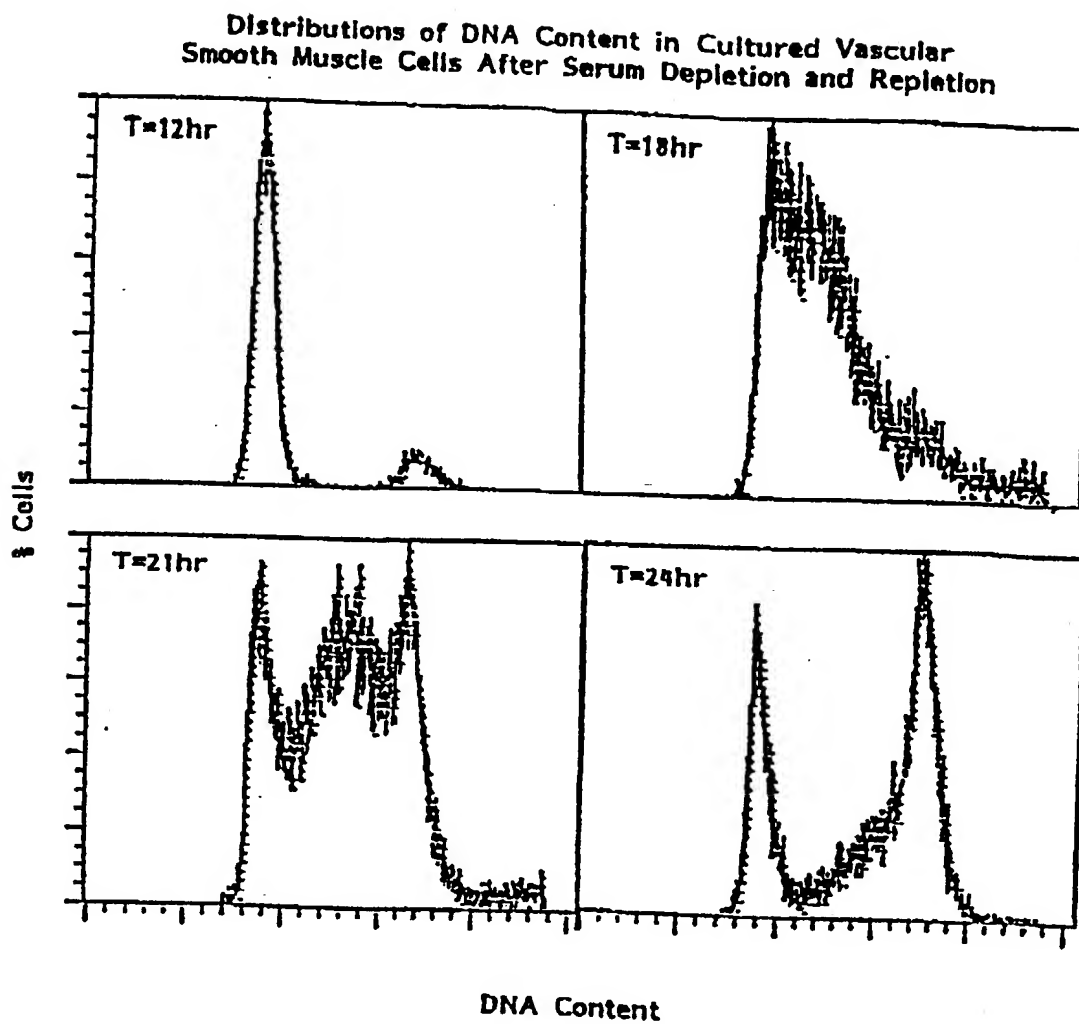


Figure 4

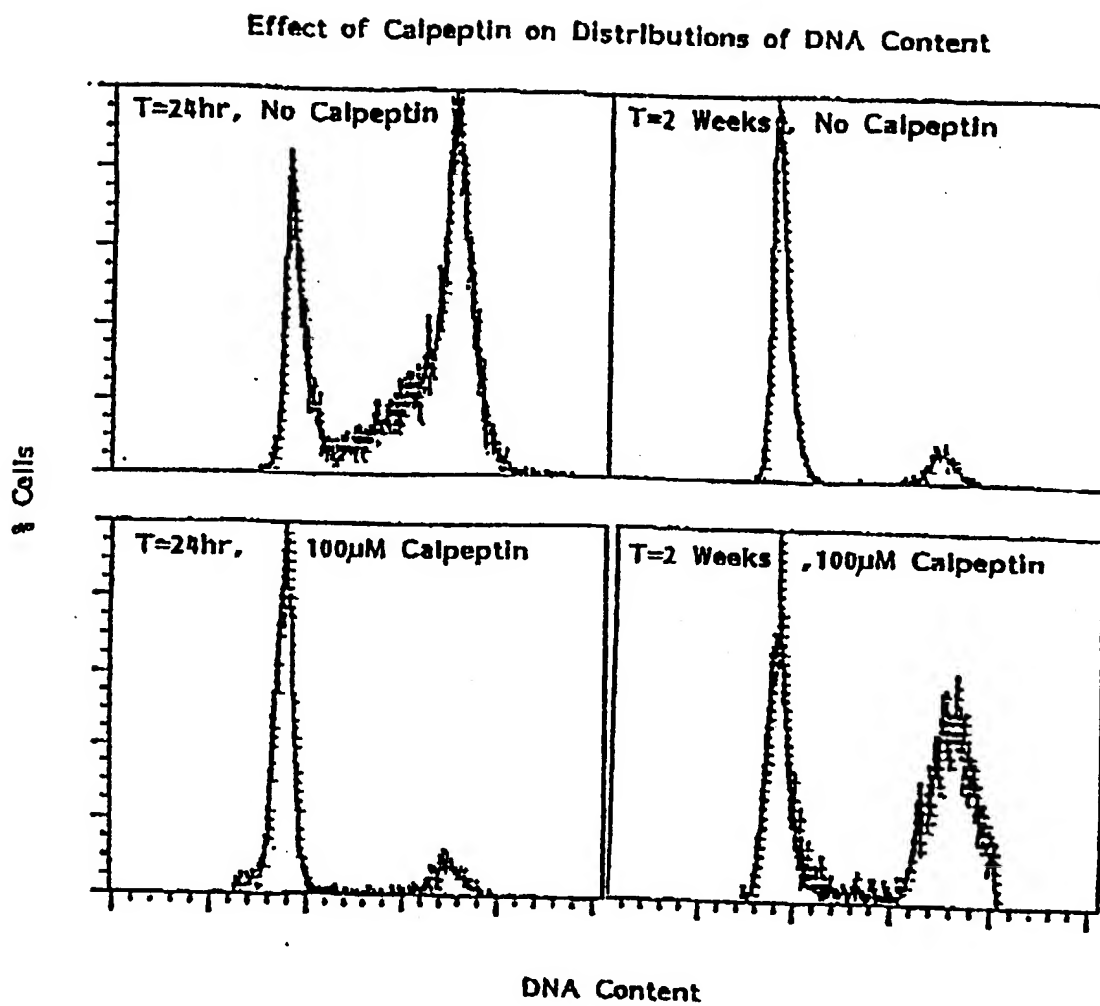
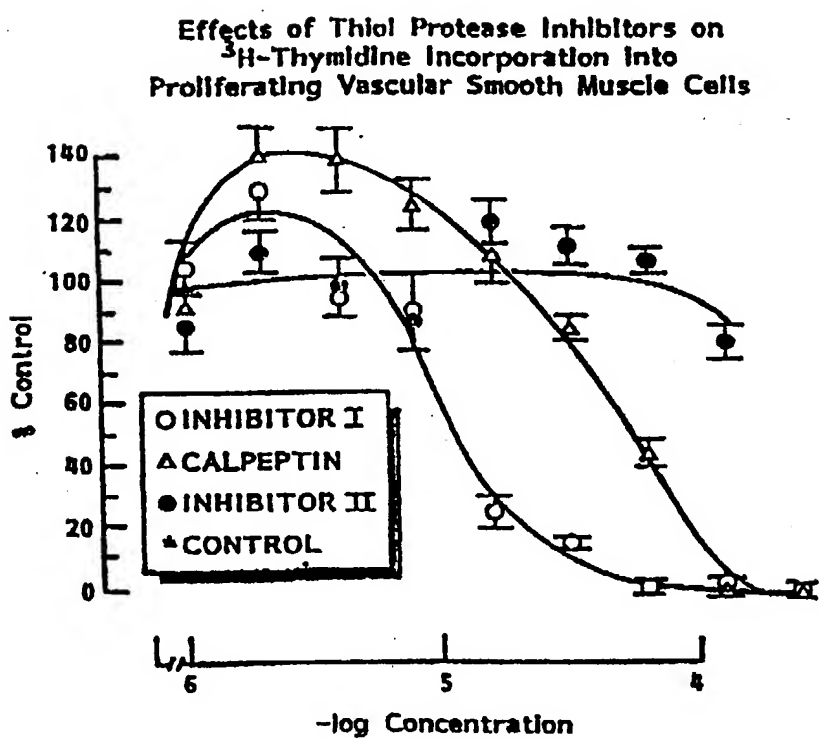


Figure 5



INTERNATIONAL SEARCH REPORT

International Application No. PCT/US92/00905

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ³		
According to International Patent Classification (IPC) or to both National Classification and IPC		
IPC (5): A61K 37/02; C07K 5/00		
US CL : 530/329,330,331; 514/17,18,19		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁴		
Classification System	Classification Symbols	
U.S.	530/329,330,331; 514/17,18,19	
Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched ⁵		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴		
Category*	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸
X/Y	US, A. 4,752,602 (Lipsky et al.) 21 June 1988, see entire document.	1,3-7,16,18/9
X/Y	EP, A. 0,383,190 (Guindon et al.) 22 August 1990, see entire document.	1,10,16,18 / 2,8,11,12,13,14,15,16-18
Y	Biochemical And Biophysical Research Communications, Volume 153, No.3, issued 30 June 1988, Tsujinaka et al. "Synthesis Of A New Cell Penetrating Calpain Inhibitor (Calpeptin)", pages 1201-1208, see tables I and II on pages 1204-1205.	16-18
Y	JP A. 61-103897 (Murachi et al.) 22 May 1986 See entire document.	16-18
Y	Blood, Volume 76, No. 12, issued 15 December 1990, Fox et al., "The Role of Calpain in Stimulus-Response Coupling: Evidence That Calpain Mediates Agonist-Induced Expression of Procoagulant Activity In Platelets", pages 2510-2519, see entire article.	16-18
<p>* Special categories of cited documents:¹⁶</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&" document member of the same patent family</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search ²	Date of Mailing of the International Search Report ²	
05 MAY 1992	21 MAY 1992	
International Searching Authority ¹	Signature of Authorized Officer ²⁰	
ISA/US	Deborah France for Bennett Celsa	

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US92/00906

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ³		
According to International Patent Classification (IPC) or to both National Classification and IPC		
IPC (5): A61K 37/02; C07K 5/00 US CL : 530/329,330,331; 514/17,18,19		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁴		
Classification System	Classification Symbols	
U.S.	530/329,330,331; 514/17,18,19	
Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched ⁵		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴		
Category ⁶	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸
X/Y	US, A. 4,752,602 (Lipsky et al.) 21 June 1988, see entire document.	1,3-7,16,18/9
X/Y	EP, A. 0,383,190 (Guindon et al.) 22 August 1990, see entire document.	1,10,16,18 / 2,8,11,12,13,14,15,16-18
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Y	JP A. 61-103897 (Murachi et al.) 22 May 1986 See entire document.	16-18
Y	Blood, Volume 76, No. 12, issued 15 December 1990, Fox et al., "The Role of Calpain in Stimulus-Response Coupling: Evidence That Calpain Mediates Agonist-Induced Expression of Procoagulant Activity In Platelets", pages 2510-2519, see entire article.	16-18
<p>* Special categories of cited documents:¹⁵</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"S" document member of the same patent family</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search ²	Date of Mailing of the International Search Report ²	
05 MAY 1992	21 MAY 1992	
International Searching Authority ¹	Signature of Authorized Officer ²⁰	
ISA/US	D. Bennett Celsa for	